EFFICIENT PRODUCTION OF MUTANT PROTEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 USC 119 to European Application No. 82902117.1 filed in the Netherlands, August 11, 1989, which disclosure is hereby incorporated by reference.

INTRODUCTION

Technical Field

This invention relates to methods and compositions for preparation of mutant proteins, particularly *Bacillus* proteases, using an homologous *Bacillus* host strain modified so that it lacks the capacity to produce a corresponding native protease.

Background

Bacilli are widely used for the production of industrially important enzymes such as α-amylases, neutral proteases and alkaline (or serine) proteases. *Bacilli* are used, at least in part, because they are capable of secreting large quantities of protein, they do not produce substances considered hazardous, and they have a long history of safe industrial use. As an indication of their safety, several species of *Bacilli* have been approved for the production of proteins intended for use in food.

There are, however, problems with the use of *Bacilli* as production hosts for recombinant proteins. They produce and secrete a variety of proteases which tend to degrade the heterologous proteins produced. Approximately 90 percent of this proteolytic activity is attributed to neutral metalloprotease (npr) and serine protease (apr). Several approaches have been used to overcome this problem, including the use of double mutants deficient in both extra-cellular neutral and

alkaline proteases and inactivation of ~~endogenous~~ intracellular protease genes. However, the organisms so obtained were not completely devoid of extra-cellular proteolytic activity.

Other approaches used to overcome the problems with *Bacilli* have included producing mutant proteases in host strains which are incapable of expressing the wild type protease. The transgenic cells obtained were asporogenous mutants while the untransformed host cells are normally sporulating. Since there is a strong correlation between the sporulation process and protease production, use of asporogenous mutants for the production of recombinant proteases is believed to be unsatisfactory. Introduction of a sporulation deficiency in a strain would be expected to result in decreased protease production. Others have attempted to obtain protease-negative strains by partial deletion of the protease gene or by insertion of another gene in the protease gene.

These approaches have several drawbacks including the following. The original gene may be reactivated: If a gene which is homologous to the inactivated chromosomal gene is introduced into the cell on a plasmid, homologous recombination can lead to reactivation of the gene. The original gene, although inactivated, may also lead to the production of partial expression products if the promoter region is still active. This is additionally disadvantageous in terms of metabolic efficiency. Further, the introduction of unwanted phenotypic traits may occur, for example, antibiotic resistance, depending on the gene inserted. There may also be plasmid instability due to homology between a genomic sequence and a part of the inserted plasmid. It is therefore of interest to obtain mutant cells for the production of mutant proteases in which all sequences with possible homology between the chromosome and the plasmid encoding a mutant protease to be inserted into the mutant cell have been deleted.

Relevant Literature

The use of Bacillus subtilis as an expression host is disclosed by Palva, et *al. Gene* (1982) 19:81-87. The expression of the α-amylase gene from *B. amyloliquefaciens* in *B. subtilis* is described, Palva *et al, supra*. See also Fahnestock and Fischer, *J. Bacteriol* (1986) 165:796-804; Schein, *et al., Biotechnology* (1986) 4:719-725; and Wang, *et al., Gene* (1988) 69:39-47 for descriptions of heterologous gene expression in *B. subtilis*.

A *B. subtilis* double mutant deficient in both extra-cellular neutral and alkaline protease is disclosed by Kawamura and Doi, *J. Bacteriol* (1984) 160:442-444. Construction of an apr *B. subtilis* strain starting from a npr strain using a plasmid vector containing the apr gene to introduce the *cat* gene both as an inactivator and a selectable marker is disclosed by Fahnestock and Fischer, *Appl. Environ. Microbiol.*, (1987) 53:379-384. Partial deletion of an additional cellulase protease gene (*epr*) from *B. subtilis* is described as showing marginal contribution to the extracellulose protease activity of the *B. subtilis* by Sloma, *et al., J. Bacteriol* (1988) 170:5557-5563. A *B. subtilis* strain incapable of secreting enzymatically active subtilisin or neutral protease but which expresses heterologous protease genes isolated or derived from *B. amyloliquefaciens* is disclosed in EP-A-0246678. The *B. subtilis* host was a normally a sporulating cell; the resulting mutants were asporogenous. *B. subtilis* with a reduced extra-cellular protease level is also disclosed in WO 86/01825. No expression and secretion of heterologous proteins is suggested. Expression of subtilisin analogs in *B. subtilis* is disclosed in WO 88/8033. A *B. subtilis* strain with low secreted protease activity is also disclosed in WO 89/04866.

Wells, *et al.*, *Nucleic Acids Research* (1983), 11:7911-7925 disclose the expression of *B. amyloliquefaciens subtilisin* BPN' in *B. subtilis* using its own transcription signals. However, Jacobs, *et al.*, *Nucleic Acids Research* (1985) 13:8913-8926 show that this strategy is not generally applicable: The 5' region of subtilisin Carlsberg derived from *B. licheniformis* did not contain functional signals for transcription in *B. subtilis*. A *B. subtilis* promoter was required.

SUMMARY OF THE INVENTION

In accordance with the subject invention, transformed *Bacillus* strains and methods for their use are provided for the efficient production of a protein of interest, particularly a mutant *Bacillus* protease. The *Bacillus* expression hosts are incapable of producing a wild-type protease and may have an asporogenous phenotype. The expression host is obtained by removing indigenous DNA sequences capable of homologous recombination with a DNA sequence encoding the polypeptide of interest from the chromosome of a parent of the expression host prior to transformation with an integration cassette comprising the DNA sequence encoding the polypeptide of interest. At least one copy of the integration vector is integrated into the chromosome of the expression host. Optionally, additional copies may be integrated into the chromosome, or be present on a plasmid. The transformed protease expression host ~~negative *Bacillus*~~ is grown to express the polypeptide of interest. The method finds particular use in the production of mutant serine proteases using a protease expression host incapable of producing wild-type protease ~~negative expression host~~ derived from an alkalophilic *Bacillus*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of plasmid pM58 containing parts of the 5′ and 3′ flanking regions of the alkaline protease gene from *Bacillus* novo sp. PB92.

Figure 2 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-1 into plasmid PM58, giving the plasmid PEM58.

Figure 3 shows the introduction by homologous recombination, of the plasmid pEM58 into the 5′ flanking region of the protease gene of *Bacillus* strain PBT110.

Figure 4 shows a simplified restriction map of the regions surrounding the deleted protease gene after illegitimate recombination. Two strains were obtained PBT125 and PBT126.

Figure 5 nucleotide sequence of the ~~hindIII~~ HindIII fragments of PBT125 (A) and PBT126 (B).

Figure 6 schematic representation of the introduction of the protease gene into M13 mp18.

Figure 7 shows the nucleotide sequence of plasmid pBHA-1.

Figure 8 shows the introduction of the protease gene of *Bacillus* strain PBT110 in plasmid pBHA1.

Figure 9 shows the reorientation of the F1 ori containing *Bam*HI fragment of pBHAI-MXL giving plasmid pBHAR-MXL.

Figure 10 shows the subcloning of the 3′ sequence of the high alkaline protease into plasmid pBHAR-MXL, giving plasmid pBHARB-MXL.

Figure 11 shows the deletion of the *E. coli* sequence from plasmid pBHARB-MXL M216Q giving plasmid pBHB-MXL M216Q.

Figure 12 shows the reorientation of the neomycin resistance gene in plasmid pE194neo-1 giving plasmid pE194neo-3.

Figure 13 schematically shows the introduction of the temperature-sensitive origin of replication from plasmid pE194neo-3 into plasmid pBHB-MXL M216Q giving plasmid pEN3Q.

Figure 14 schematically shows the construction of strain *Bacillus* strain PEP111.

Figure 15 schematically shows the construction of stain *Bacillus* strain PEP211.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, *Bacillus* expression hosts, particularly asporogenous alkalophilic *Bacillus* expression hosts, which are protease negative are provided for the preparation of a polypeptide of interest. Naturally occurring DNA and synthetic genes may be employed for the production of the polypeptide of interest. Methods of obtaining production of the polypeptide of interest, especially in homologous hosts, include the steps of obtaining a mutant *Bacillus* which is incapable of producing native proteases, transforming it with an integration cassette comprising a DNA sequence encoding the polypeptide of interest and growing the resulting transformed cell to express the polypeptide of interest. These protease-negative strains find particular use in the production of mutant proteases.

Bacilli offer several advantages as expression hosts. They are capable of producing large quantities of protein and are approved for the preparation of protein designated for human consumption. The use of protease negative *Bacilli* offers the additional advantage that the protein produced will be not be degraded by endogenous extracellular proteolytic activity. Further, where the expression host is derived from the same or a related strain as the original source of the DNA encoding the polypeptide of interest, for example a mutant serine protease, transcriptional and translational initiation and termination regulatory

regions native to the gene to be expressed may be used, which facilitates obtaining expression and which permits expression of the polypeptide of interest at levels at least substantially similar to those of the native protease in the untransformed parent cell. Where protease negative alkalophilic *Bacilli* are used as the expression hosts, they offer the additional advantage that the risk of contamination with other microorganisms is minimized due to the alkaline pH of the growth medium during fermentation, which is not well tolerated by other organisms.

Rendering the expression host incapable of producing indigenous proteases necessitates the replacement and/or inactivation of the wild-type gene from the genome of a parent of the mutant *Bacillus* strain cell. The mutation is preferably a non-reverting mutation. Various methods can be used. For example, one method is to clone the gene or part thereof, modify it by site-directed mutagenesis and reintroduce the (partial) gene into the cell on a plasmid. By homologous recombination the (partial) gene may be introduced into the chromosome. The result is that the wild-type and the mutant gene are located in tandem on the chromosome. After a second recombination, the modified sequence is left in the chromosome having thereby effectively introduced the mutation into the chromosomal gene. Furthermore all other plasmid sequences are deleted

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Another method which can be used is to inactivate the chromosomal gene copy by deletion. Preferably, the chromosomal gene fragment that is deleted is the complete coding region, the deletion having occurred in such a way that reversion becomes impossible. The deletion may be a partial deletion, provided that the sequences left in the chromosome are too short for homologous recombination with a plasmid-encoded protease gene. More preferably the gene is completely deleted from the chromosome of an unaltered parent of an expression host. Following inactivation or

deletion, the DNA sequence of interest, which can be a mutant copy of the inactivated gene, is transformed into the cell on a cloning vector using methods well known to those skilled in the art. The protease negative strain obtained can be used advantageously for the expression of both homologous and heterologous proteins. The expression of mutant proteases using as an expression host a protease negative derivative of the strain from which the wild-type gene was isolated is considered to be homologous expression.

Deletion of the indigenous protease gene can be carried out as follows. A wild type protease gene including its 5′ and 3′ regions is isolated and inserted into a cloning vector. The coding region of the protease gene is then deleted from the vector *in vitro*, leaving behind a sufficient amount of the 5′ and 3′ flanking sequences to provide for homologous recombination with ~~an~~ the indigenous protease gene in the unaltered parent host. The vector is then transformed or transfected into the parent *Bacillus* strain. The vector is integrated into the chromosome via homologous recombination in ~~the flanking region.~~ one of the flanking regions. Outrecombination and resolution leads to a *Bacillus* strain in which the protease gene has been deleted.

The vector used is preferably a plasmid. To provide for selection of transformants, a selectable marker is included in the plasmid, for example, antibiotic resistance such as neomycin resistance. Additionally, the vector preferably is one which can be selectively integrated into the chromosome. This can be achieved by introducing an inducible origin of replication, for example a temperature sensitive origin such as that obtainable from pE194, into the plasmid. By growing the transformants at a ~~temperature~~ temperature to which the origin of replication is sensitive, the replication function of the plasmid is inactivated, thereby providing a means for selection for chromosomal integrants; integrants may be ~~identified~~ selected for by

growth at high temperatures in the presence of the relevant antibiotic (e.g. neomycin).

Resolution of the plasmid from the host chromosome can leave the flanking regions in the chromosome while removing the coding region. Finally, the resulting mutant selected for has lost the selectable marker gene (is neomycin sensitive) and is protease negative. The final chromosomal configuration is determined by restriction analysis and Southern blotting. Possible integration mechanisms are extensively described in WO 88/06623. Integration by the so-called Campbell-type mechanism can take place in the 5' flanking region of the protease gene, resulting in a protease positive strain carrying the entire plasmid vector in its chromosome in the protease locus. Since illegitimate recombination will give different results it will be necessary to determine whether the complete gene has been deleted. To obtain more specific strains, homologous recombinants also could be made. The protease gene, modified by site-directed mutagenesis, is then introduced into the protease negative cell on a suitable expression vector. Care is taken that the plasmid carries short or preferably no sequences homologous to the expression host chromosome to avoid plasmid ~~instability it is desired to maintain the plasmid.~~ instability.

Integration of the mutated protease gene into the chromosome of the protease negative strain can be achieved by leaving the flanking regions of the protease gene bordering the mutated protease gene in the plasmid. Introduction of such a plasmid can give rise to homologous recombination in the flanking regions thereby introducing the mutated protease gene in the chromosome of the protease negative strain. This is especially advantageous if the plasmid is unstable. The present invention also shows that the amount of recombinant mutant or wild-type protease obtained is comparable or even higher if one copy of the gene is integrated

in the genome than if there are several copies of the gene present which are encoded by a plasmid.

Yet another method of inactivating the native protease is to mutagenize the chromosomal gene copy. Specific mutations *in vivo* may be obtained after transformation of bacteria with oligonucleotides which are mutagenic. Alternatively, the chromosomal protease gene can be replaced with a mutant gene by homologous recombination, without prior isolation of a protease negative strain.

Of particular interest in the present invention is the production of proteases derived from alkalophilic *Bacilli*. These proteases are referred to herein as high alkaline proteases. As a host cell for the expression of mutated genes encoding modified or so-called protein engineered proteins it is preferable to use cells in which the genes are structurally expressed at a high level. Therefore, for the production of high alkaline proteases, preferably alkalophilic *Bacillus* strains are used as host cells.

High alkaline protease producing *Bacilli* are taxonomically not well classified and are generally referred to as alkalophilic *Bacillus* strains. For the present invention alkalophilic bacilli are defined as *Bacillus* strains that grow under alkaline conditions, generally pH 9-11 (Horikoshi, and Akiba, (1982), *Alkalophilic microorganisms*, Springer Verlag, New York). The alkaline proteases produced by such *Bacilli* are called high alkaline proteases. Examples of *Bacillus* strains capable of growing at alkaline pH are described in, for example, U.S. Patent Nos. 3,723,250, Re. 30,602 and 4,480,037.

For large scale production of proteases strains of *Bacilli* capable of growing under conditions typically used for commercial enzyme production are preferred. Such strains, sometimes referred to as "Industrial strains" originate from organisms obtainable from various sources. They may be isolated from the soil or

are available from depositories or other sources and are obtained by genetic modification of such *Bacillus* strains. Industrial *Bacillus* strains are defined in EP-A-0134048. They are characterized as follows. They are resistant to genetic exchange, such as phage infection or transformation. The strains are stable and may or may not be capable of spore formation. They are usually prototrophic and have been modified to provide for high yields of endogenous protein products, such as the enzymes alpha-amylase and various proteases under commercial fermentation conditions. The yield of an endogenous protein product obtained in an industrial production process can amount to at least 5 g/l (0.5% w/v) of secreted protein. Industrial strains typically also secrete DNases, which can result in degradation of DNA in the medium, providing for protection against genetic exchange, but also render the cells resistant to transformation.

Of particular interest as expression hosts are strains that are not subject to catabolic repression. Induction or repression controls the expression of many extracellular enzyme operons and removal of the requirement for an inducer can result in high yields as well as avoiding the expense of adding inducers to industrial fermentations. Further, organisms that are insensitive to catabolic repression often synthesize and secrete extracellular enzymes earlier in the growth cycle, thus improving production efficiency. See, for example, Aspects of Microbiology (1984) 9: 54-55. Commercial growing conditions, for optimum cost effectiveness use strains which are capable of growing to high cell densities without causing high viscosity, are able to grow on cheap raw materials, are genetically stable, are phage resisant and are not prone to cell lysis. By using an industrial protease producing *Bacillus* strain as a host for the production of the mutants of this protease, the high efficiency seen for production of the original protease is also obtained

with the mutant protease. This results in a surprisingly superior production efficiency compared to laboratory strains. One chromosomal copy of the mutated gene gives upon expression and secretion in an industrial strain generally results in a yield even higher than 50 plasmid copies containing the mutated gene, in a laboratory strain.

An example of an alkalophilic *Bacillus* host strain is *Bacillus* novo species PB92 disclosed *inter alia* in U.S. Patent No. Re. 30,602. Derivatives of these alkalophilic *Bacillus* strains, that have been optimized for protease production, are employed to produce their proteases on an industrial scale (see EP-A-0284126). The products are used in several industrial applications e.g., as an additive in laundry detergents. Examples of such products are Maxacal^{R} (Gist-brocades/IBIS), savinase^{R} (NOVO), Esperase^{R} (NOVO). Mutants of such products have been described in WO 89/06279, where they are described as being derived from *Bacillus lentus* strains, and in the not prepublished European Patent Application EP-A-0328229.

It is an aspect of this invention that mutant proteases can be produced efficiently by a homologous *Bacillus* strain by way of exchanging the chromosomal gene or a part thereof with the corresponding mutated gene. In this way the production capacity for the wild-type protease is eliminated while at the same time introducing production capacity for a mutant protease. In another aspect of the present invention it was surprisingly found that asporogenous *Bacilli* can be used to obtain high level expression of the protease gene. A preferred group of asporogenous mutant *Bacillus* strains are those derived from PBT110 and its derivatives. The polypeptide of interest may be any polypeptide for which expression is desired and may be either homologous (derived from the host cell) or heterologous (derived from a foreign source or a synthetic DNA sequence). Preferably the polypeptide is a mutant high

alkaline protease, most preferably the mutant protease is one described in EP-A-0328229 and WO89/06279 and capable of production on an industrial scale. The DNA sequences encoding the polypeptide of interest can be partially or totally synthesised using conventional techniques, or isolated from natural sources using techniques well known to those skilled in the art. Where it is desired to obtain a mutated protease, the DNA sequence can be mutated so that at least one amino acid is different from the wild type protease. More than one amino acid may be mutated so long as the resulting peptide maintains the capacity to degrade substitutes ~~substitue~~. In some instances the reaction rate may be lower than that of the native protease; or it may be the same or greater reaction rate as native protease, depending upon the desired application.

Once the desired DNA sequence has been obtained it may be manipulated in a variety of ways to provide for expression. After each manipulation of the DNA in the development of an integration vector, the plasmid will be cloned and isolated and as required the particular vector component analysed as to its sequence to ensure that the proper sequence has been obtained.

Where the gene of interest is to be expressed in an expression host which recognises the natural transcriptional and translational regulatory regions of the gene of interest, the entire gene, following mutation as desired, with its natural 5′ and 3′-regulatory regions may be introduced into an appropriate expression vector. However, where the gene is to be expressed in a host which recognizes the natural transcriptional and ~~transcriptional~~ translational regulatory regions less well, further manipulation may be required. The non-coding 5′ region upstream from the gene of interest may be removed by ~~removed by~~ endonuclease restriction, Bal31 resection, or the like. Alternatively, where a convenient restriction site is present near the 5′-terminus of the gene of interest, the gene of interest may be restricted and an

adapter employed for linking the gene of interest in proper reading from with a promoter region functional in the host cell.

To obtain high production of a high alkaline protease obtainable from *Bacillus* PB92 in *B. subtilis* it is preferred to insert a promoter which is active in *B. subtilis* in front of the gene of interest. If enzymes derived from other *Bacilli* are produced in *B. subtilis* insertion of a promoter active in *B. subtilis* or other expression signals may be necessary.

The plasmid containing the gene of interest may be included within a replication system for episomal maintenance in the expression host or may be provided without a replication system where it may become integrated into the expression host genome. The DNA may be introduced into the expression host in ~~accordand~~ accordance with known techniques. Transformation of alkalophilic *Bacillus* strains will preferably involve the use of protoplasts from said strains. However, the conventional protoplast transformation protocol as described by Chang and Cohen, *Molec. Gen. Genet. 168* (1979) 111-115, does not work for alkalophilic *Bacillus* strains. The protocols needed for these strains have been disclosed in EP-A-0283075, which is herein included by reference.

Once the gene of interest has been introduced into the expression host, the host may be grown to express the gene of interest. The expression host may be grown to high density in an appropriate nutrient medium. Where the promoter is inducible, permissive conditions may be employed, for example, temperature change, exhaustion or excess of metabolic product or nutrient, or the like. Preferred growth conditions are commercial production conditions such as broth comprising inexpensive raw materials and absence of inducers, and conditions which allow for high cell density to be achieved.

To obtain the expression product, where the expression product is retained in the host cell the cells are harvested, lysed and the product isolated and purified by extraction, precipitation, chromotography, electrophoresis and the like. Where the product is secreted into the medium, the nutrient medium may be collected and the product isolated by conventional means, for example affinity chromatography. Since, in the described expression host the wild-type protease gene has been completely deleted, the expression product preparation is completely free from the wild-type protease. This is particulary desirable for ease of purification where the expression product is a mutant protease, especially a homologous mutant protease.

Where the expression product is a protease, after purification it can be formulated into a detergent composition. The high alkaline proteases in particular find use in detergent compositions and in laundry processes.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

Construction of Inactivation Plasmid pEM58Δ

Plasmid pM58 (see EP-A-0284126 which disclosure is hereby incorporated by reference) was digested with restriction enzymes *Bal*I and *Hpa*I. The large fragment containing part of the flanking sequences of the protease gene was purified (Maniatis, Molecular cloning: A Laboratory Manual, Cold Spring Harbor (1982)) and ligated. The ligation mix was transformed (Spizizen *et al.*, *J. Bacteriol.* 81 (1961) 741-746) to *Bacillus subtilis* DB104 (Kawamura and Doi, *J. Bacteriol.* 160 (1984) 442-444). Neomycin resistant and protease negative transformants were selected for on minimal plates containing 0.4% casein and 20 μg/ml neomycin.

The resulting plasmid pM58Δ was isolated and characterized by restriction enzyme analysis. As shown in Figure 1, plasmid pM58Δ contains the flanking sequences of the alkaline protease gene, the gene coding for neomycin resistance and a *Bacillus* origin of replication.

To construct an integration plasmid containing a temperature sensitive origin of replication, pM58Δ was digested with restriction enzymes *Xba*I and *Bgl*II. The fragment containing the alkaline protease flanking sequences was purified and ligated to the purified *Xba*I/*Bgl*II fragment of pE194neo-1 (EP-A-0284126) containing the temperature sensitive origin of replication, derived from pE194 (Jordanescu *et al.*, *Plasmid* (1978) 1: 468-479) after the fragment had been purified as described. The ligation mix was transformed to *B. subtilis* DB104 and transformants having neomycin resistance were selected for (see above). Plasmid pEM58Δ (see Figure 2) was isolated and characterized. It contains the neomycin resistance gene, the temperature sensitive origin of replication from pE194 and the flanking sequences of the alkaline protease gene. The resulting plasmid pEM58Δ was used to transform *Bacillus* strain PBT110.

EXAMPLE 2
Construction of a Protease Negative Alkalophilic Bacillus Strain

Protoplast transformation of Bacillus PBT110 by plasmid pEM58Δ

Bacillus strain PBT110 is an asporogenous mutant of *Bacillus* novo species PB92 (U.S. Patent No. Re. 30,602) and was obtained using classic UV mutation procedures. The PBT110 was transformed with plasmid pEM58Δ using a method similar to that described in EP-A-0284126. Prior to integration experiments, transformants were checked by restriction enzyme

analysis to verify that they contained the relevant plasmid.

Integration of pEM58Δ in to the Bacillus PBT110 chromosome

Integration of pEM58Δ into the chromosome of *Bacillus* strain PBT110 was performed as described in EP-A-0284126. Selection for integrants was carried out at a neomycin concentration of 1 μg/ml. The genetic organization of the integrant was determined by restriction enzyme analysis followed by Southern blotting and hybridization analysis. The results are shown in Figure 3. Integration of pEM58 apparently took place through the so-called Campbell-type mechanism by homologous recombination in the 5' flanking region of the alkaline protease gene. The resulting *Bacillus* strain is PBT110-INT5.

Selection for protease negative strains

Bacillus strain PBT110-INTS was inoculated in 100 ml tryptic soya broth (TSB) containing 1 μg/ml neomycin and incubated for 24 hours at 50°C. After 24 hours, 0.1 ml of the so culture obtained was inoculated in a 500 ml shake flask containing 100 ml PBT minimal medium: K_2HPO_4, 17.42 g/l; glutamate, 13.36 g/l; citrate.H_2O 2 g/l; $FeSO_4.7H_2O$, 0.05 g/l; $ZnSO_4.7H_2O$, 1 mg/l; $MnSO_4.H_2O$, 1 mg/l; $CaCl_2.2H_2O$, 10 mg/l; $MgSO_4.7H_2O$, 0.2 g/l; $CuSO_4.5H_2O$, 0.5 mg/l; H_3BO_3, 0.5 mg/l; $Na_2MoO_4.2H_2O$, 0.5 mg/l; $CoCl_2.6H_2O$, 0.5 mg/l; Biotine, 0.5 mg/l; saccharose, 20 g/l. The pH was adjusted to pH 8.0, then the medium was sterilized for 20 min at 120°C. After sterilization 1 mg/l thiamine was added.

The culture was incubated for 4 days at 37°C. After 4 days, 1 ml of culture was diluted in 100 ml of the same medium and incubated for 4 days. After 4 days the culture was plated on PBT minimal medium plates containing additionally 0.4% casein and 15 g/l agar. Colonies which lacked a detectable protease halo were

considered protease minus. These protease negative colonies were then analyzed to verify the absence of the gene encoding the PBT110 alkaline protease.

Characterization of protease negative strains

Strains which did not show a detectable halo on the casein plates described in the previous section were primarily tested for neomycin sensitivity and asporogenous phenotype. Two strains, *Bacillus* PBT125 and PBT126, which contained the protease negative and asporogenous phenotype were tested for protease production in 500 ml shake flasks containing 100 ml protease production medium, as described in U.S. Patent No. Re. 30,602. Neither strain produced detectable amounts of protease.

The genetic organization of the two strains was determined by restriction enzyme analysis and chromosomal blotting. A simplified restriction map of the regions surrounding the deleted protease gene after illegitimate recombination is shown in Figure 4. The two strains had complete deletion of the protease and neomycin resistance genes. A small plasmid fragment of undefined size appeared to be left in the chromosome after deletion of the protease gene. The sequence of this fragment was determined as follows. The chromosomal *Hin*dIII fragments of strains PBT125 and PBT126 containing the plasmid/chromosome junctions were ligated in phage vector M13 mp18 (Messing *et al.*, *Nucl. Acids Res.* (1981)9: 303-321) and transfected to *E. coli* JM101 according to the procedure described by Cohen *et al.*, Proc. Natl. Acad. Sci. (USA) (1972) 69: 2110-2114. After phage propagation in *E. coli* JM101, ssDNA was isolated (Heidecker *et al.*, *Gene* (1980) 10: 69-73). The inserts were sequenced using the method described by Sanger *et al.*, *Proc. Natl. Acad. Sci. (USA)* (1977) 74: 6463. The nucleotide sequences of the *Hin*d III fragments are shown in Figures 5A and 5B, respectively for PBT125 and PBT126.

EXAMPLE 3

Construction of Protease Production and Mutation Vectors

Subcloning of the PB92 protease gene in phage M13 mp18

Plasmid pM5-8 (Stanssens et al *Nucleic Acids Res.* (1987) 17: 4441-4484) was digested with *Hpa*I and *Bal*I. After purification of the DNA fragment containing the protease gene (Maniatis, (1982) supra) this fragment was ligated into phage M13 mp18 which was digested with *Sma*I. The ligation mixture was transfected to *E. coli* JM101 (Cohen et al., supra). After phage propagation in *E. coli* JM101, dsDNA was isolated according to the method described by Birnboim and Doly (*Nucl. Acid Res.* (1979) 7: 1513-1523). The insert and its orientation were checked by restriction enzyme analysis. Vector mp18MXL was used for further subcloning experiments: In Figure 6 is shown in the introduction of the protease gene into M13MP18.

Subcloning of the PB92 protease gene in plasmid pBHA1

In Figure 7 is show the nucleotide sequence of plasmid pBHA-1. This plasmid is derived from the twin vector system pMa/c5-8 described by Stanssens et al., (*Nucleic Acids Res.* (1989) 17: 4441-4454) into which an additional promoter has been inserted. Plasmid BHA1 consists of the following fragments:

pos 11-105: bacteriophage FD, terminator;
pos 121-215: bacteriophage FD, terminator;
pos 221-307: a part of plasmid pBR322 (viz. positions 2069-2153);
pos 313-768: bacteriophage F1, origin of replication (viz. pos 5482-5943);
pos 772-2571: part of plasmid pBR322 (viz. the origin of replication) and the β-lactamase gene;
pos 2572-2685: transposon Tn903, complete genome;

pos 2719-2772: tryptophan terminator (double);
pos 2773-3729: transposon Tn9, the chloramphenicol-acetyltransferase (*cat*) gene (the nucleotides at pos 3005(A), 3038(C), 3302(A) and 3409(A) differ from the wild-type *cat* coding sequence. The mutations were introduced to eliminate the *Nco*I, *Bal*I, *Eco*RI and *Pvu* II sites);
pos 3730-3804: multiple cloning site;
pos 3807-7264: part of plasmid pUB110 (viz. the replication function and kanamycin resistance gene, *Eco*RI-*Pvu*II fragment) (McKenzie *et al.*, *Plasmid* (1986) 15: 93-103 and *Plasmid* (1987) 17: 83-85);
pos 7267-7331: multiple cloning site.

The fragments were combined using known cloning techniques, e.g., filling in sticky ends with Klenow, adapter cloning, etc. All data were derived from Genbank^R National Nucleic Acid Sequence Data Bank (NIH), U.S.A. Plasmid pM5-8 was deposited under DSM 4566.

Modification of Plasmid for Mutagenesis

Prior to mutation procedures the twin vector system pBHA/C-1 was modified to obtain the twin vector system pBHARB-MXL/pBHCRB-MXL, by the following method. Vector mp18-MXL (see above for preparation) was digested with *Kpn*I and *Hinc*II. The DNA fragment containing the protease gene was purified and ligated into pBHA1 which was digested with *Eco*RV and *Kpn*I. The ligation mixture was transformed to E. coli JM101 (Maniatis, (1982) *Supra*) and plated on LC$^+$ plates containing 10 g/l trypton (Difco) 5 g/l yeast extract (Difco); 8 g/l NaCl; 25 g/l thymine; 1 g/l $MgSO_4$ $7H_2O$; 100 µg/ml ampicillin; and 20 µg/ml neomycin; pH 7.0. Plasmid DNA from the transformants was isolated (see Birnboim, *supra*) and characterized by restriction

enzyme analysis. In this way pBHAl-MXL was isolated (see Figure 8).

In order to sequence mutations introduced in the protease gene with an available set of oligonucleotides, it was necessary to reverse the orientation of the F1 origin compared to the protease gene. This was performed in the following way. Plasmid pBHAl-MXL was digested with *Bam*HI and religated. The ligation mix was transformed to *E. coli* WK6 (Maniatis, (1982) *supra*) and selected for neomycin or ampicillin resistance on LC^+ plates containing 10 g/l trypton; 5 g/l yeast extract (Difco); 8 g/l NaCl; 25 mg/l thymine; 1 g/l $MgSO_{7H_2O}$, 100 µg/ml ampicillin; and 20 µg/ml neomycin pH 7.0. Plasmid DNA of transformants was isolated (Birnboim, *supra*) and characterized by restriction enzyme analysis. In this way pBHAR-MXL was isolated, which differs from pBHA-MXL in that the orientation of the *E. coli* sequence is reversed, (*see* Figure 9).

For the introduction of additional flanking sequences in plasmid pBHAR-MXL, the following digestions were made. Plasmid pM58 was digested with *Sph*I and *Hin*dIII. Plasmid pBHAR-MXL (Figure 10) was digested with *Sph*I and *Hin*dIII. The larger fragment was purified. Both digests were ligated and transformed to *B. subtilis* DB104 and selected for neomycin resistance and protease activity on minimal plates containing 10 µg/ml neomycin and 0.4% casein. Transformants were characterized by restriction enzyme analysis. One of these, pBHARB-MXL (*see* Figure 10) was used for further experiments.

Mutagenesis of the PB92 gene in plasmid DBHARB-MXL

Mutagenesis was carried out with the twin vector system pBHARB-MXL/pBHCRB-MXL obtained as described above. A method based on the gapped-duplex approach (Kramer et al., Nucl. Acids Res. (1984) 12: 9441) and a ~~phlasmid~~ plasmid (phage/plasmid hybrid) was used. A phasmid is

a combination of phage and a plasmid containing all the information needed to clone or express genes or other DNA sequences. Essentially the method rests on a gapped-duplex DNA intermediate consisting of a gapped strand (- strand) containing a wild-type antibiotic resistance marker and a template strand (+ strand) carrying an amber mutation in the gene conferring resistance to the antibiotic. After annealing, the mutagenic oligonucleotide becomes incorporated in the gapped strand during *in vitro* gap-filling and sealing reaction. The resultant molecules are used to transform a mismatch repair deficient (MutS) host in which the linkage between the intended mutation and the antibiotic resistance marker is preserved. The mixed plasmid population, isolated from this strain, is then allowed to segregate in a suppressor negative host strain. Transformants are plated on antibiotic containing medium, thus imposing a selection for progeny derived from the gapped strand. Two plasmid vectors were isolated: In the pMa type vector, nucleotide 3409 is changed from G to A, while in the pMc type vector nucleotide 2238 is changed from G to C, creating amber stop codons in the chloramphenicol-acetyltransferase gene and β-lactamase gene, respectively, rendering these genes inactive.

To perform mutagenesis the target DNA fragment is cloned into the multiple cloning site of pMa5-8 or a derivative thereof. A gapped duplex between pMa5-8 containing the target DNA and pMc5-8 is then constructed. The single strand gap, consisting of the target DNA, may be subjected to mutagenesis with a mutagenic oligonucleotide having long synthetic oligonucleotides and a low level of misincorporated nucleotides obtained, using chemical or enzymatic misincorporation of nucleotides. For a detailed description, *see* Ausubel *et al.*, (1987), *Current Protocols in Molecular Biology*, (John Wiley & Sons Inc. New York); or B. Perbal, (1988), *A Practical Guide to*

Molecular Cloning, (2nd ed.) (John Wiley & Sons Inc; New York.).

Construction of Vectors for Industrial Protease Production

After the introduction of the desired mutation(s) in plasmid pBHARB-MXL, the unwanted *E. coli* sequences from the plasmid were deleted as follows: plasmid pBHARB-MXL containing the relevant mutation was digested with BamHI and religated under diluted low DNA concentration conditions. The ligation mixture was transformed to *B. subtilis* DB104. Neomycin resistance and protease activity were selected for on minimal plates containing 20 μg/ml neomycin and 0.4% casein. The DNA of the transformants then was isolated and characterized by restriction enzyme analysis. In this way vectors were isolated which lack *E. coli* DNA and thus suitable for commercial production of proteins.

This procedure is illustrated in Figure 11 using as example the use of mutation M216Q to construct in plasmid pBHB-MXL M216Q. M216Q, and also M216S, S160D and N212D referred to hereinafter, are mutant proteases of *Bacillus* PB92, and described in EP-A-0328229

EXAMPLE 4

Protoplast Transformation of Bacillus PBT125 by Plasmid pBHB-MXL or Derivatives Thereof

Bacillus strain PBT125 was transformed with plasmid pBHB-MXL, or plasmids derived therefrom containing mutant protease genes, following the transformation procedure described in EP-A-0284126. Prior to production studies, restriction enzyme analysis of the transformants was performed to verify that they contained the relevant plasmid.

EXAMPLE 5

Construction of Protease Integration Vectors

Integration vector pEN_3Q

Plasmid pE194neo-1 (EP-A-0284126) was digested with *Sal*I and religated. The ligate was transformed to *B. subtilis* DB104. Transformants having neomycin resistance were selected for on minimal plates containing 20 µg/ml neomycin. Plasmid pE194neo-3 was isolated from one of the transformants and the presence of the neomycin resistance gene in a reversed orientation verified ~~how?~~ (see Figure 12). Plasmid pE194neo-3 was digested with *Hpa*I and *Bgl*II. A fragment containing the temperature sensitive origin of replication was detained and purified (Maniatis, (1982) *supra*).

Plasmid pBHB-MXL M216Q (see Example 3 and Figure 11) was digested with *Bgl*II and *Bal*I. A fragment containing the protease gene was purified. The fragments containing the temperature sensitive origin of replication and the protease gene were ligated. The ligation mix was transformed to *B. subtilis* DB104. Transformants having protease activity and neomycin resistance were selected for as described above. Transformants also were characterized by restriction enzyme digestion. One transformant, containing plasmid pEN_3Q, was selected (Figure 13). This plasmid contained the neomycin resistance gene, the temperature sensitive origin of replication from plasmid pE194neo-3 and the M216Q mutation in the PB92 protease gene.

Integration vector pEN_3S.

In the same way as described for integration vector pEN_3Q, pEN_3S was constructed using pBHARB-MXL M216S as the starting vector.

EXAMPLE 6

Construction of Bacillus Strains PEP111 and PEP112 Containing a Single Mutant Protease Gene

Protoplast transformation of Bacillus PBT110 by Plasmid pEN_3Q

Bacillus strain PBT110 was transformed with plasmid pEN_3Q as described in EP-A-0284126. Prior to integration experiments, it was checked by restriction enzyme analysis to verify that the transformants contained the relevant plasmid.

Integration of pEN_3Q in to the Bacillus PBT110 chromosome

Experiments to integrate plasmid pEN_3Q in to the chromosome of *Bacillus* strain PBT110 were performed as described in EP-A-0284126. Selection for integrants was carried out at a neomycin concentration of 1µg/ml at nonpermissive temperatures for plasmid replication (50 °C). To verify that pEN_3Q had integrated into the chromosome, chromosomal DNA of potential integrants was isolated, digested with *Hin*dIII or *Cla*I, run on a 0.8% DNA agarose gel and blotted to nitrocellulose (Southern, J. Mol. Biol. (1975) 98: 503-517) and hybridized with ^{32}P labeled nick-translated pEN_3Q DNA (Maniatis, (1982) *supra*). Integration of pEN_3Q occurred by homologous recombination, resulting in a strain (PT110/Q) with two protease genes (one wild type and one mutant M216Q) tandemly located on the chromosome. Thus, integration of pEN_3Q occurred by a so-called Campbell-type mechanism as depicted in Figure 14 (see also EP-A-0284126).

Selection of neomycin sensitive recombinants

Neomycin sensitive recombinants were selected, which contained either the wild-type or the mutant protease gene in the chromosome (hereinafter called

"outrecombinants"). The selection procedure is similar to the procedure described above in Example 2C.

Strain PBT110 M/Q (obtained as described above) was used as the starting strain. After strain PBT 110 M/Q was incubated in PBT minimal medium (37°C for 3 x 24 hours at 250 rpm), the culture was plated on PBT minimal medium plates containing 0.4% casein and 15 g/l agar. These plates were incubated for 48 h at 37 °C then replica-plated to Heart infusion (HI) plates containing either 1 µg/ml neomycin or no neomycin. Those colonies which were Neomycin sensitive were considered outrecombinants.

Characterization of neomycin sensitive recombinants.

Chromosomal DNA of putative outrecombinants was isolated, digested with *Cla*I or *Hin*dIII, run on a 0.5% aqarose gel, blotted to nitrocellulose (Southern, (1975) *supra*) and hybridized with ^{32}P-labeled nick translated pEN$_3$QDNA (Maniatis, (1982) *supra*). Since mutation M216Q results in the removal of a *Cla*I site, the strains containing a wild-type protease or a mutant protease gene, and the intermediate strain PBT110 M/Q can be easily distinguished, (*see* Fig. 14).

An outrecombinant containing the M216Q mutant protease gene of PB92 was isolated. After it was shown that a gene replacement of PB92 wild-type protease to PB92 mutant protease had occurred, the product of the strain was characterized by its biochemical parameters (k_{at}, K_m) oxidation resistance, specific activity and wash performance, as described in EP-A-0328229 The results indicated that for these parameters, the mutant protease had the same or similar characteristics as control protease M216Q, indicating that the product of the transformed strain was indeed the PB_{92} protease containing the mutation M216Q. This transformed strain is called *Bacillus* PEP111.

Similar experiments were performed using vector pEN_3S to construct *Bacillus* strain PEP112, which contains the PB_{92} protease with mutation M216S.

EXAMPLE 7

Construction of Bacillus strains PEP211 and PEP212 Containing Two Mutant Protease Genes

Bacillus strain PEP111 (obtained as described above) was transformed with plasmid pEN_3Q, following the procedure described in EP-A-0284126. Prior to the integration procedure, restriction enzyme analysis was used to verify that the transformants contained the relevant plasmid. The integration procedure and the selection for integrants was performed as described in EP-A-0284126 using a neomycin concentration of 20 μg/ml and growth at non-permissive temperatures for plasmid replication (50 °C).

To verify that plasmid pEN_3Q had integrated in to the chromosome, chromosomal DNA of putative integrants was isolated and digested with *Hin*dIII, run on 0.8% agarose gels, blotted to nitrocellulose (Southern et *al.* (1979) *supra*) and hybridized with ^{32}P-labeled nick translated pEN_3Q. A strain containing two mutant (M216Q) PB_{92} protease genes separately located on the chromosome was isolated. This strain is called *Bacillus* PEP211. Its chromosomal organization is shown in Figure 15.

To obtain an analogous strain containing two protease genes with mutation M216S, strain PEP112 containing a mutant gene (M216S) after gene replacement of the wild-type gene (See Example 6, above), was transformed with plasmid pEN_3S as described above for PEN3Q. *Bacillus* PEP212 containing two separately located PB_{92} mutant M216S protease genes on the chromosome was obtained.

EXAMPLE 8

Production of Mutant Proteases

The transformed *Bacillus* strains in which the mutated protease encoding gene was either localized on a plasmid or on the chromosome were used for producing mutant proteases. For plasmid-encoded production two types of strains were used, the alkalophilic strain *Bacillus* PBT125, as described herein, and *B. subtilis* strain DS12367, an asporogenic strain derived from *B. subtilis* DB104 (see Kawamura *et al.*, *J. Bacteriol.*(1984) 160:442-444). These strains were transformed with pBHB-MXL derived plasmids. *Bacillus* PBT125 and *B. subtilis* DS12367, which do not have protease encoding plasmids, were used as control strains. For production with strains containing chromosomally integrated protease mutant genes the *Bacillus* PEP strains, as described herein, were used. *Bacillus* PBT strains 108 and 110, containing the wild-type protease genes were used as control strains. PBT108 is a strain containing two wild-type genes separately located on the chromosome, see EP-A0284126. For PBT110, see USPN Re.30,602.

To verify that the original promoter of the PB_{92} protease gene is functional in *B. subtilis,* a construct was made, pBHB-MXLR, in which the orientation of the protease gene to the *Hpa*II promoter (Zyprian *et al.*, *DNA* (1986) 5: 219-225) was reversed, so that the expression of the protease gene was directed only by its original promoter.

Analysis of protease production by the various strains were performed in 500 ml shake flasks containing 100 ml protease production medium (see U.S. Patent No. Re. 30,602) with the following changes, depending upon the strain used. When alkalophilic *Bacillus* strains were used the cultures were incubated, after inoculation, for 40 hours at 37°C in a production medium as described in U.S.P.N. Re. 30,602. In the case of plasmid containing-strains, neomycin (20 µg/ml) was added to the production medium. When *B. subtilis* DS12367 strains were used, a comparable medium containing 12.5 g/l yeast extract (Difco); 0.97 g/l

$CaCl_2.6H_2O$; 2.25 g/l $MgCl2.6H_2O$; 20 mg/l $MnSO4.4H_2O$; 1 mg/l $CoCl.6H_2O$; 0.5 g/l citrate; 0.5 ml antifoam 5693 (Basildon) 6% w/w maltose; 0.2 M phosphate buffer pH 6.8 was used. In the case of plasmid-containing strains, neomycin (20 g/ml) was added to the medium.

Cultures containing each of the *B. subtilis* strains were incubated for 65 hours at 37°C, with shaking. In all cases, the shake-flasks were inoculated with 0.1 ml of a tryptic soya broth culture containing 20 μg/ml neomycin containing the relevant strain which had been incubated for 24 hours at 37°C.

Protease activity was assayed using dimethylcasein as substrate as described by Lin *et al.*, *J. Biol. Chem.* (1969) 244: 789-793. The specific activities of the protease mutants (EP-A-0328229) were used to determine the production of protease per mg. of protein.

The results of the fermentation experiments are summarized in the following Table 1.

Table 1

Strain	Relative protease production (%)	Mutation
PBT110	100	WT
PBT125	0	--
PBT108	120	WT
PEP111	100	M216Q
PEP112	100	M216S
PBT125 pBHB-MXL	95-100	WT
PBT125 pBHB-MXL M216Q	95-100	M216Q
PBT125 pBHB-MXL M216S	95-100	M216S
PBT125 pBHB-MXL S160D	95-100	S160D
PBT125 pBHB-MXL N212D	95-100	N212D
PEP211	120	M216Q
PEP212	120	M216S
DS12367	0-1	--
DS12367 pBHB-MXLR	4	WT
DS12367 pBHB-MXL	40	WT
DS12367 pBHB-MXL M216Q	40	M216Q
DS12367 pBHB-MXL M216S	40	M216S
DS12367 pBHB-MXL S160D	40	S160D
DS12367 pBHB-MXL N212D	40	N212D

It is evident from the above results that mutant proteases may be obtained in high yield in Bacilli from when the corresponding unmodified protease gene has been deleted. The *Bacilli* used for expression of the mutant proteases are preferably alkalophilic *Bacillus* strains, more preferably alkalophilic *Bacillus* which are capable of producing at least 5 g/l (0.5% w/v) protein under conditions which are used for commercial enzyme production. Use of a homologous *Bacillus* for production of the mutant proteases offers several advantages, including an expression capacity of the host cell for the mutant protease at least substantially similar to that of native gene product.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.